Diversity of kresoxim-methyl sensitivities in baseline populations of *Venturia inaequalis*

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Abstract: Several strobilurin fungicides inhibiting fungal respiration by binding to cytochrome *b* have been introduced recently. A mechanism of strobilurin resistance identified as active in several plant pathogenic fungi is based on the activation of alternative respiration. Thus far, respective studies have been restricted to single isolates of respective pathogens. Here, we report a study on 250 *Venturia inaequalis* baseline isolates to the strobilurin kresoxim-methyl having a broad sensitivity distribution characterized by a 50-fold difference in sensitivity of the most- and least-sensitive isolates. For the majority (62%) of these isolates, differences in sensitivity were not caused by the interference of alternative respiration with the full inhibitory potency of kresoxim-methyl. Rather, variable dose-responses with largely different degrees of inhibition achieved at a low dose of kresoxim-methyl were found to be responsible. For 38% of the baseline isolates, alternative respiration was already active during the stage of conidia germination. Activation of this pathway was, again, dependent on the strobilurin dose. Selection of sub-populations of isolates resisting low doses of kresoxim-methyl by multiple mechanisms and the recombination among isolates expressing such mechanisms singly can be expected to be slowed by an anti-resistance strategy based on high strobilurin doses.

Keywords: alternative respiration; kresoxim-methyl; salicylhydroxamic acid; SHAM; strobilurins; *Venturia inaequalis*; apple scab

1 INTRODUCTION

Kresoxim-methyl (BAS 490F) and trifloxystrobin (CGA 279202), both members of the novel class of strobilurin fungicides, have been introduced for the control of apple scab caused by *Venturia inaequalis* (Cooke) Winter.[1–3] All natural and synthetic strobilurins act as inhibitors of respiration by binding to the center Q_P of cytochrome *b* of the mitochondrial bc_1 complex.[4–13] During mycelial stages of development, several fungal pathogens have been found to be considerably less sensitive to strobilurins than expected from the intrinsic potencies of respective inhibitors.[5,12–18] This mechanism of mycelial resistance has been explained by the circumvention of the cytochrome *b* target site through the induction of alternative respiration.[12–18] Although spore germination was identified as a stage highly sensitive to strobilurins,[1,6] a laboratory mutant of *Septoria tritici* Rob.[10] and an orchard isolate of *V inaequalis*[18] have been described as exceptions. Here, alternative respiration was already active during the germination of spores. In both cases, however, this mechanism of strobilurin resistance active under in-vitro test conditions had no adverse impact on the protective activities of strobilurins in the control of respective diseases.[10,18]

The apparent discrepancy between strobilurin potencies under in-vitro and in-vivo test conditions implies that the pathway of alternative respiration indigenous to plants and fungal organisms[12–21] is not necessarily active during infectious stages of plant pathogens. A model explaining this apparent lack of alternative respiration during host invasion was presented for *Magnaporthe grisea* (Hebert) ME Barr causing rice blast disease.[13] According to the rice blast model, secondary plant products such as flavones will scavenge active oxygen species known to be involved in the induction of alternative oxidase, the functional core of alternative respiration.[16,19–21] In compliance with this model, flavone strongly synergized the inhibition of mycelial growth of *M grisea* by metomistrobin (SSF 126)[13] and of *V inaequalis* by kresoxim-methyl.[18]

Thus far, the impact of alternative respiration on strobilurin sensitivities of plant pathogens during different developmental stages has been studied with a single or a few isolates of plant pathogenic fungi. A study describing kresoxim-methyl sensitivities expressed by single isolates of a large baseline population of *V inaequalis* was presented recently.[22] Here, we analyze the impact of alternative respiration and other mechanisms on the sensitivities of these baseline isolates to kresoxim-methyl during conidia germina-

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tion under in-vitro conditions. The stage of conidia germination was chosen because both kresoxim-methyl and trifloxystrobin were described as primarily active in a protective mode of apple scab control.[1–3]

2 EXPERIMENTAL

2.1 Fungal isolates and materials

As described by Olaya and Köller,[22] conidia were produced from mycelia of 250 monoconidial isolates representing five orchard populations across all major apple-growing regions in North America. Mycelial colonies were grown on potato dextrose agar (PDA), and conidia of individual isolates were produced on PDA covered with cellophane as described previously.[18,22]

Kresoxim-methyl (technical) was obtained from BASF Corporation (Research Triangle Park, NC). PDA was from Difco Laboratories (Detroit, MI). All other chemicals were from Sigma Chemical Company (St Louis, MO).

2.2 Tests of kresoxim-methyl sensitivities of *Venturia inaequalis* isolates

Kresoxim-methyl was dissolved in acetone and salicylhydroxamic acid (SHAM) was dissolved in methanol. Water was amended with inhibitor solutions adjusted to the inhibitor concentrations to be tested, with solvent concentrations not exceeding 3 ml liter^{-1}. Effects on conidia germination at these solvent concentrations were negligible.

The sensitivity of germinating conidia was tested with 30 µl of a conidial suspension (10^5 conidia ml^{-1}) in water containing kresoxim-methyl at a dose of 0.01 µg ml^{-1}, reflecting the typical ED_{50} value of a typical isolate,[18,22] or at a 10-fold higher dose of 0.1 µg ml^{-1}. SHAM was tested at a dose of 100 µg ml^{-1}, either alone or in mixture with kresoxim-methyl. This dose of SHAM was chosen because synergistic effects on kresoxim-methyl have been shown to be complete at this dose.[18] At this dose of SHAM, germination of conidia was not, or only slightly (<20%), affected for 70% of the isolates tested; for the remainder, inhibition was 20–50%.

The sensitivity of germinating conidia was tested as described previously.[18,22] In brief, suspensions of conidia were placed on the polystyrene surface of Petri dishes and incubated for 24 h at 20 °C. Conidia germination was assessed for 100 conidia at each inhibitor concentration, with conidia rated as germinated if a normally developing germ tube had at least the length of a conidium or had formed an appressorium.

Isolate sensitivities were expressed as relative germination (RG) defined as:

$$RG = \frac{\text{percentage germination in presence of inhibitor}}{\text{percentage germination in absence of inhibitor}} \cdot 100$$

When mixtures of kresoxim-methyl and SHAM were tested, germination in the presence of SHAM alone served as the non-treated control. The mean standard error inherent to the test at a single inhibitor dose, determined for several isolates, was RG = 7 (± 3).[22]

2.3 Data analysis

Mean RG values were compared by the nonparametric Kolmogorow–Smirnov test as employed previously.[23,24] Antagonistic, additive or synergistic effects of SHAM on kresoxim-methyl activities were calculated by applying the formula

$$Exp = X + Y - XY/100$$

described by Richter,[25] with *Exp* as the percentage of inhibition expected from additive effects, and with X and Y as the percentages of inhibition obtained with SHAM and kresoxim-methyl tested singly. If the inhibition observed for the mixture (*Obs*) exceeded the expected additive effects (*Exp*), the interaction was rated as synergistic. In order to allow for variances caused by imprecisions inherent to the test, interactions were rated as fully synergistic if the difference $Obs - Exp$ was $> 15\%$.

3 RESULTS

3.1 Effects of SHAM on isolate sensitivities of germinating conidia to kresoxim-methyl

As described recently,[22] kresoxim-methyl sensitivities of germinating conidia determined for 250 baseline isolates of *V inaequalis* were broadly distributed, with ED_{50} values ranging from 0.003 µg ml^{-1} to 0.14 µg ml^{-1}. The impact of alternative respiration on this broad distribution of sensitivities was tested with the alternative oxidase inhibitor SHAM.[10,14,18–20] When SHAM was tested in mixture with 0.01 µg ml^{-1} kresoxim-methyl, the population became more sensitive than in the absence of SHAM (Fig 1). The mean RG in the presence of SHAM was 59 as compared to 68 in its absence. This sensitizing effect of SHAM was highly significant ($P < 0.001$); it was reflected in

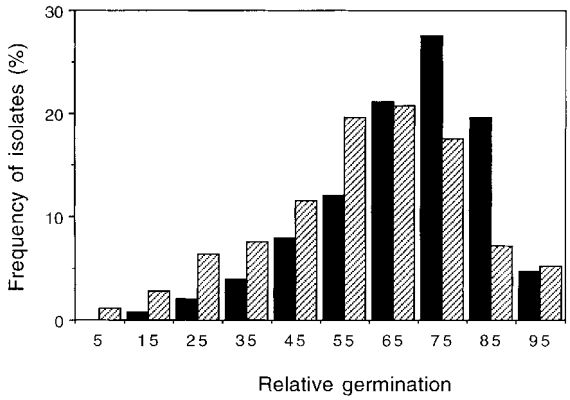

Figure 1. Frequency distribution of response of *Venturia inaequalis* isolates to kresoxim-methyl. Conidia were produced from 250 isolates, and the in-vitro sensitivity was determined in (■) absence and (▨) presence of salicylhydroxamic acid (SHAM). Inhibitor concentrations were 0.01 µg ml^{-1} for kresoxim-methyl and 100 µg ml^{-1} for SHAM. For the mixture, germination in the presence of SHAM alone served as the non-treated control.

higher frequencies of isolate sensitivities in the range of RG < 60 and lower frequencies for isolates falling into the range of RG = 71–90. The frequencies of least-sensitive isolates (RG > 90), however, were identical under both test conditions (Fig 1).

The interactive effects of SHAM on individual isolates were not homogenous for the 0.01 μg ml^{-1} test dose of kresoxim-methyl. As shown in Fig 2, effects ranged from slightly antagonistic (*Obs* − *Exp* < 0%) to additive (*Obs* − *Exp* = 0%), slightly synergistic (*Obs* − *Exp* ⩾ 15%) and fully synergistic (*Obs* − *Exp* > 15%). There was no correlation ($r = 0.2$) between inhibitory effects of SHAM and interactive effects (*Obs* − *Exp*), indicating that synergistic/antagonistic effects of SHAM are independent from its inhibitory effects and that the lack of synergism observed for the majority of isolates was unlikely to have been caused by insufficient uptake of the alternative oxidase inhibitor.

At a 10-fold higher dose of kresoxim-methyl (0.1 μg ml^{-1}), conidia germination was fully inhibited (RG = 0) for 53% of the isolates; the least-sensitive isolate displayed an RG value of 72 (Table 1). All isolates were fully inhibited from germination when SHAM was present in respective tests (Table 1). For isolates displaying RG values > 20 in the absence of SHAM (15%), SHAM effects were fully synergistic (*Obs* − *Exp* > 15%).

3.2 Population structure of conidia responses to kresoxim-methyl

Analysis of the sensitivity data obtained for germinating conidia in the absence or presence of SHAM allowed us to distinguish four groups of *V inaequalis* phenotypes (Table 2): isolates on which SHAM had no synergistic effect (Group 1), isolates synergized at either the high (Group 2) or the low (Group 3) dose of kresoxim-methyl, and isolates synergized at both inhibitor doses (Group 4).

Group 1 of isolates was not affected by alternative respiration at either kresoxim-methyl dose and comprised the majority of isolates (62%) (Table 2). At the low strobilurin dose of 0.01 μg ml^{-1}, however, RG values tended to be broadly distributed and ranged from 17 to 93, very similar to that for the entire population (Fig 1). This broad range of sensitivities was not reflected in isolate responses to the 10-fold higher kresoxim-methyl dose; here, the majority of group 1 isolates (72%) was fully prevented from germination (RG = 0) (Table 2). The discrepancy between a broad range of inhibition (7–83%) observed at the 0.01 μg ml^{-1} dose and an almost complete inhibition achieved at the 10-fold higher dose indicated that the steepness of dose-responses was accountable for by different isolate responses to kresoxim-methyl.

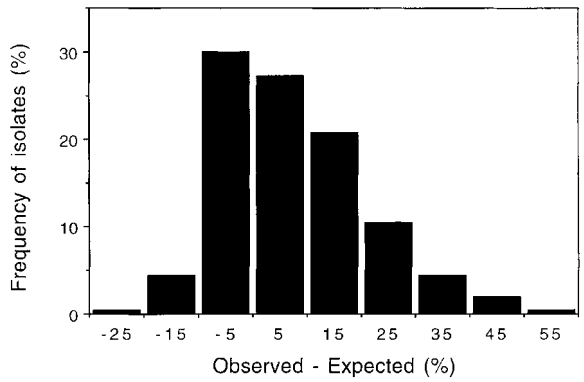

Figure 2. Interactive effects of salicylhydroxamic acid (SHAM) on the inhibition of conidia germination by kresoxim-methyl. Conidia germination was tested in-vitro for 250 baseline isolates of *Venturia inaequalis*. The percentages of inhibition calculated for additive effects of inhibitor action (Expected)[25] were subtracted from percentages of inhibition observed in the presence of both inhibitors (Observed). Inhibitor concentrations were 0.01 μg ml^{-1} for kresoxim-methyl and 100 μg ml^{-1} for SHAM.

Table 1. Effects of SHAM[a] on isolate sensitivities of germinating conidia of *Venturia inaequalis* to kresoxim-methyl tested at a dose of 0.1 μg ml^{-1}

	Number of isolates	
RG[b]	*−SHAM*	*+SHAM*
0	133	250
1–20	79	0
21–40	27	0
41–60	9	0
>60	2	0

[a] Salicylhydroxamic acid at a dose of 100 μg ml^{-1}.
[b] Relative germination.

The possibility of such variable dose-responses was tested by correlating RG values obtained at the 0.01 μg ml^{-1} inhibitor dose with the differences of RG values at both doses ($RG_{0.01} - RG_{0.1}$) as a measure of dose-response steepness. The significant correlation ($r = 0.97$) obtained for all isolates (Fig 3A) was largely due to the fact that the majority of group 1 isolates were fully inhibited at the high dose of kresoxim-methyl. However, the same correlation was obtained for isolates with incomplete inhibition (RG > 10) at the high kresoxim-methyl dose of 0.1 μg ml^{-1} (Fig 3B). The result indicated that the steepness of dose responses was indeed variable among the isolates of group 1.

For group 2 of isolates, comprising 14% of the isolates tested, SHAM exerted no synergistic effects at the low strobilurin dose of 0.01 μg ml^{-1} (Table 2). Mean RG values at this dose were similar in the absence and presence of SHAM, but the mean was significantly ($P < 0.001$) higher than for the non-synergized group 1 of isolates (Table 2). Moreover, there was no correlation between RG values determined at the low dose of kresoxim-methyl and the differences obtained at both doses ($RG_{0.01} - RG_{0.1}$) ($r = 0.3$). This lack of correlation was accompanied by a high mean RG value obtained at the 0.1 μg ml^{-1} dose (Table 2). In the presence of SHAM, however, inhibition at this high dose was complete, indicating that interference of alternative respiration with full

Table 2. Variable responses to kresoxim-methyl of conidia of *Venturia inaequalis* isolates germinating in the absence and presence of SHAM

		0.01 μg ml^{-1} kresoxim-methyl					0.1 μg ml^{-1} kresoxim-methyl		
		−SHAM		+SHAM[a]			−SHAM		+SHAM
Isolate group	n	RG mean	RG range	RG mean	RG range	P[b]	RG mean	RG range	RG mean
1	156	62.2	17–93	59.9	3–93	0.32	2.1	0–18	0
2	35	77.2	63–93	76.5	51–94	0.83	32.3	21–69	0
3	50	76.4	38–95	41.8	3–70	<0.001	4.1	0–15	0
4	9	81.1	62–92	54.9	35–72	<0.001	29.9	21–72	0

[a] Salicylhydroxamic acid (SHAM) was tested at a dose of 100 μg ml^{-1}.
[b] Comparison of mean RG values obtained with 0.01 μg ml^{-1} kresoxim-methyl in the absence and presence of SHAM.

inhibitory potencies of kresoxim-methyl was not yet induced at the low, but activated at the high, dose of the strobilurin (Table 2).

Group 3 of isolates (20%) was distinguished by alternative respiration already active at the low inhibitor dose of 0.01 μg ml^{-1}, as indicated by the significantly different mean RG values determined in the absence and presence of SHAM (Table 2). In the absence of SHAM, the mean RG value was significantly ($P < 0.001$) higher than for group 1 isolates; in the presence of SHAM, the mean RG values became significantly lower, indicating that this group 3 of isolates displayed the highest intrinsic sensitivities to kresoxim-methyl. Very similarly to group 1, germinating conidia were highly sensitive to the higher test dose of 0.1 μg ml^{-1} even in the absence of SHAM (Table 2). Group 4, as the smallest group of isolates (4%), was synergized by SHAM at both kresoxim-methyl doses.

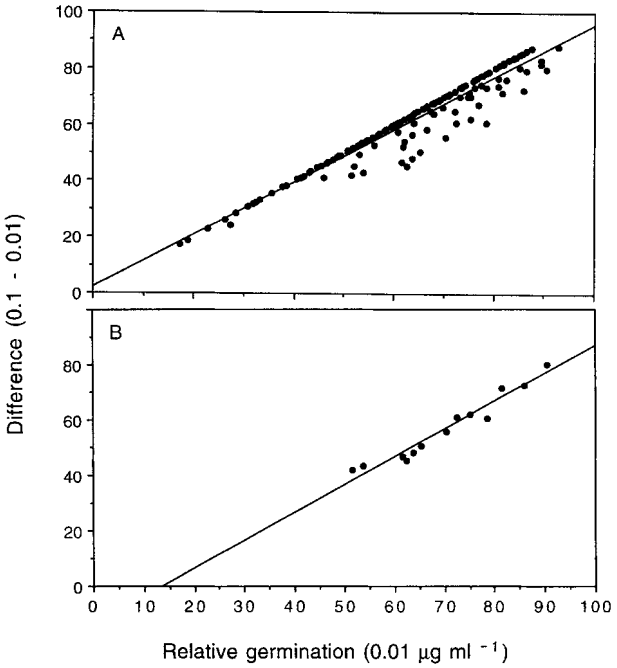

Figure 3. Dose-responses to kresoxim-methyl of the group 1 (Table 2) of *Venturia inaequalis* isolates not affected by alternative respiration. RG values determined for conidia germinating in-vitro in the presence of 0.01 μg ml^{-1} for kresoxim-methyl were regressed against the difference of RG values (Difference (0.1 − 0.01)) determined for kresoxim-methyl at concentrations of 0.1 μg ml^{-1} and 0.01 μg ml^{-1}. **A**, All isolates ($n = 156$). **B**, Isolates with RG values > 10 at 0.1 μg ml^{-1} kresoxim-methyl ($n = 13$).

4 DISCUSSION

Induction of alternative respiration, allowing continued respiration in the presence of high strobilurin doses, has been identified as the reason for unexpectedly low strobilurin sensitivities of fungal mycelia.[12–18] Recent studies have indicated, however, that interference of alternative respiration with strobilurin action is not restricted to this stage and can already be active during the stage of spore germination.[10,18] For example, alternative respiration was responsible for a 60-fold difference of sensitivities to kresoxim-methyl determined for two arbitrarily chosen isolates of *V inaequalis*.[18]

A similarly broad range of sensitivities of germinating conidia to kresoxim-methyl was found for a large baseline population of *V inaequalis*.[22] The most sensitive isolate identified in the study displayed an ED_{50} value of 0.003 μg ml^{-1}. Interestingly, this ED_{50} value coincided with the I_{50} values reported for the binding of kresoxim-methyl to cytochrome *b* in submitochondrial particles prepared from *Saccharomyces cerevisiae* Meyer ex Hansen and several other species and genera.[6] Thus, the ED_{50} value of the most sensitive isolate of *V inaequalis* described here appears to reflect the full and undisturbed inhibitory potency of kresoxim-methyl. The ED_{50} value determined for the least-sensitive isolate of *V inaequalis* was 50 times higher.[22]

Analysis of the impact of alternative respiration on kresoxim-methyl potencies revealed that the variability of isolate sensitivities was not exclusively explained by the known circumvention mechanism of the strobilurin target site. For the majority (62%) of isolates tested, SHAM exerted no synergistic effects on kresoxim-methyl employed at a low dose, in spite of a largely variable degree of inhibition achieved (7–83%). Isolate responses were more uniform at a 10-fold higher dose of kresoxim-methyl, suggesting that the largely variable steepness of dose-responses was accountable for different responses of isolates. At low strobilurin doses, inhibition of the cytochrome *b* target site appears to be largely variable among phenotypes of *V inaequalis*. The as yet unknown mechanism reponsible for this phenomenon is apparently overcome at higher doses of the inhibitor.

For the remaining 38% of isolates, alternative respiration antagonized the full expression of strobilurin potencies. Within this group of isolates, however, activation of the alternative pathway was again dependent on the strobilurin dose. The pathway was either activated at a low and sublethal dose of kresoxim-methyl, or it required a higher dose to be active. This apparent variability within a large population suggests that the regulation of alternative oxidase induction as the key component of alternative respiration is less uniform than might have been anticipated from results obtained with single isolates.[8,10,12,14–16,18] Regardless of a variable induction of the alternative respiratory pathway, the range of sensitivities at a low dose of kresoxim-methyl was broad even in the presence of the alternative oxidase inhibitor SHAM. This result suggests that factors other than levels of alternative oxidase remain involved in determining the sensitivity to strobilurins.

As expected from our previous results,[18] the induction of alternative oxidase during the stage of conidia germination was not a rare trait of baseline isolates. For fungal organisms, the role of alternative oxidase and the mechanism of its regulation remain largely unexplored. As reported for *Neurospora crassa* Shear & Dodge, assembly of alternative respiration is dependent on the alternative oxidase gene and modulated by a second gene not yet characterized; evidence for a family of closely related alternative oxidases as described for plants[20,21] was not apparent.[21] In *M grisea*, an alternative oxidase gene was induced in the presence of metomistrobin or hydrogen peroxide.[16] Another not-yet-characterized protein involved in increasing transcription levels over levels expressed constitutively was also identified under these conditions. A post-translational regulation of the alternative oxidase characterized for *M grisea*[26] was excluded because the two cysteine residues involved in this mechanism reported for plants[20,21,27] were lacking.[16] In contrast, a post-translational regulation very similar to plant alternative oxidases has been described for *Gaeumannomyces graminis* (Sacc) Arx & Oliv. var *tritici* Walker.[17]

Regardless of questions to be resolved, experimental evidence exists that the mechanism of strobilurin resistance based on alternative respiration observed under in-vitro conditions is of low or no impact during infectious stages of respective pathogens including *V inaequalis*.[10,18,22] According to a model derived from *M grisea*,[13] secondary plant products such as flavones act as scavengers of active oxygen involved in the induction of alternative oxidase. Although the inhibition of conidial germination by kresoxim-methyl occurs on plant surfaces, conidia escaping the purely protective action of the inhibitor by activating alternative respiration could still be controlled at a subcuticular location, even in the presence of scavenging flavones. Surface systemic and translaminar movement of kresoxim-methyl has been discussed.[2] Although the current evidence appears to suggest that an alternative respiration mechanism is unlikely to be of importance in the development of practical strobilurin resistance, phenotypes restricting such natural oxygen scavengers from penetration into fungal cells are likely to exist. A potential mechanism of preventing natural plant products as well as DMI fungicides from entering fungal cells has been described recently.[28,29] Isolates capable of restricting the uptake of natural scavengers of active oxygen would continue to induce alternative oxidase and to antagonize strobilurin action during infectious stages.

A second phenomenon of practical concern and superimposed on the impact of alternative respiration on strobilurin potencies is the largely variable response of isolates determined at low inhibitor doses. Under practical low-dose situations (eg low application rates and long spray intervals), isolates of *V inaequalis* belonging to the least-sensitive part of the sensitivity distribution can be expected to be selected through a population response very similar to the DMI fungicides and dodine.[23,24] Although declining performances could be counteracted with higher doses of strobilurins once such populations resistant to low strobilurin doses were selected, tactics of disease management would have to be revised accordingly. As expected from the sample size analyzed, indications for a mutational change of the cytochrome *b* target site giving rise to highly resistant isolates was not apparent. Such target site mutations have been described for several fungi,[30–33] but experimental data allowing assessment of this risk are lacking at present.

In summary, sensitivities of *V inaequalis* isolates to kresoxim-methyl analyzed within a population context revealed a broad and continuous distribution of isolate sensitivities similar to those reported for DMI fungicides and dodine.[23,24] In these cases, practical resistance was based on the selection of the least-sensitive part of isolates already detectable in baseline populations. Our analysis of kresoxim-methyl sensitivities suggested the presence of at least two mechanisms involved in the broad range of kresoxim-methyl sensitivities: (a) the surprisingly variable response to a low dose of kresoxim-methyl not relating to alternative respiration and (b) a variable dose-response apparent for the activation of alternative respiration. Although alternative respiration appears of low relevance under practical conditions of disease control, genetic recombination of isolates resisting low doses of strobilurins by either one of the mechanisms might give rise to a more resistant sub-population containing both genes involved. As evident from the analysis of baseline sensitivities to kresoxim-methyl, an anti-resistance strategy based on high strobilurin doses can be expected to slow down both the selection of respective sub-populations and the recombination among isolates expressing either one of the mechanisms. A similar high-dose strategy has been recommended for DMI fungicides.[34] Such high-dose strategies would not necessarily slow the selection of highly strobilurin-resistant mutants containing a mu-

tated target site. The relative risk inherent to this putative mechanism of target site resistance remains to be determined.

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